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Characterization of *Streptococcus pneumoniae* isolates pre- and post-vaccine introduction in Brazil

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Characterization of *Streptococcus pneumoniae* isolates pre- and post-vaccine introduction in Brazil

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2016

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An abstract of
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Abstract

Characterization of *Streptococcus pneumoniae* isolates pre and post vaccine introduction in Brazil

By Marissa Fraire

Introduction: It is estimated that around half a million children under the age of 5 die of pneumococcal disease every year worldwide. In Latin America, pneumonia is one of the leading causes of death and hospitalization for children under 5 years and in the elderly over 60 years. In 2010, the 10-valent pneumococcal conjugate vaccine (PCV) was established in Brazil's National Immunization Program. The purpose of this thesis was to evaluate any changes in serotype, genotype, and antibacterial resistance after PCV introduction in Brazil.

Methods: We used a total of 476 randomly selected invasive pneumococcal disease (IPD) isolates from Brazil that were obtained as part of the Global Pneumococcal Sequencing project; 236 of the isolates were collected in the pre-vaccination period (2008-2009) and 240 were collected post-PCV instruction period (2012-2013).

Results: A total of 47 serotypes were identified. Overall, the five most prevalent serotypes pre-PCV, accounting for >60% of IPD isolates, were 14 (29.7%), 6B (11.9%), 3 (7.6%), 19A (6.4%), and 23F (5.9%), and post-PCV introduction were 14 (9.2%), 19A (8.8%), 3 (8.75%), 12F (4.58%), and 6A (4.58%), accounting for ~35% IPD isolates. Significant decreases were observed in two vaccine-type serotypes: 14 (p-value <0.0001) and 6B (p-value<0.0009). Overall, 85.1% of the isolates were resistant to $\geq$1 antibacterial drug; however, following PCV introduction, the proportion was 81.3%. Non-susceptibility (NS) against penicillin, meropenem, and ceftriaxone declined significantly after PCV introduction. There was a strong correlation between the presence of pilus genes and specific clonal complexes, with the majority of isolates belonging to either CC156 or CC236.

Conclusions: PCV introduction has been associated with a significant decrease (p-value <0.0001) in PCV10/13 serotypes. Ongoing surveillance in Brazil will allow monitoring for the continued evolution of the pneumococcal population, and measure vaccine impacts on serotype and antibacterial resistance. Understanding these changes could help guide the development of future vaccines and prevention strategies.

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Table of Contents

List of Tables:

List of Figures:

Chapter 1: Introduction

Streptococcus pneumoniae is a Gram-positive bacterium with over 90 capsular serotypes. However, only 11 serotypes were considered to cause the majority of invasive pneumococcal disease (IPD) in children, globally, before the introduction of pneumococcal conjugate vaccines (PCV)[1,2]. Pneumococcal disease is one of the most common vaccine-preventable bacterial infections. Pneumococcal-associated illnesses can vary in severity and include sinusitis, otitis media, as well as diseases associated with mortality such as pneumonia, meningitis, and bacteremia, which are all serious forms of pneumococcal disease[1,3,4]. Children under 5 and adults over 65 years old are considered to be high risk groups[1].

Pneumococci are often carried asymptomatically in the nasopharynx [5]. Globally, children have the highest carriage rates, around 40-60% when compared with older children and adolescents [5]. This commensal pathogen is primarily spread by respiratory droplets through coughing, sneezing, or close contact with an infected person[5,6].Within the first 24 months of life, around 95% of children have been colonized by *S. pneumoniae* and 73% had obtained at least two serotypes [7]. There are many factors that can place an individual at risk for IPD: host factors include age[5,8], malnutrition[5], ethnicity, respiratory viral co-infection, lack of pneumococcal vaccination, HIV infection and other causes of immunodeficiency, and co-morbid disease such as cardiac disease or diabetes [5]. Environmental factors are also known to play a role at increasing risk: winter months, passive smoking, areas where overcrowding occurs, like day care centers[8], orphanages[9] and densely populated urban slums for example. Some indigenous populations, like American Indians, and New Zealand Maoris are at increased risk for IPD. This may be due to an excess of host and environmental risk factors discussed above.

While during the pre-antibiotic era, pneumococcal disease was recognized as a major killer in North America and Europe, and developing pneumococcal vaccines was a priority. However, the drive to prevent IPD diminished once highly effective antibacterial therapy became available. Concerns and investigations about antibacterial drug resistance surfaced with the discovery of penicillin-resistant pneumococci in South Africa in the late 1970s, and the prevalence and scope of resistance to a variety of antibacterial classes has continued to rise [10]. Soon after penicillin resistant pneumococci emerged, resistance to macrolides such as erythromycin followed[11]. About one third of *S. pneumoniae* strains were resistant to penicillin in the early 2000s[12]. Multidrug resistance (MDR), resistance to >3 antibacterial drugs, has also been a growing concern. From 1994 to 2007, almost 25% of *S. pneumoniae* were MDR in the United States[13]. In South Africa, a country with a high prevalence of penicillin resistance in children, found that about 42% of penicillin resistant strains were also resistant to trimethoprim-sulfamethoxazole[14]. In 2013, the Centers for Disease Control and Prevention released a report with the leading 18 drug-resistant threats in the United States, which included drug-resistant *S. pneumoniae* as a serious threat[15].. Some of the major factors contributing to antimicrobial resistance (AMR) today are antibacterial overuse, spread of resistant strains, and lack of vaccine availability[12].

During colonization pneumococcal genetic material can be exchanged and strains can potentially gain virulence and antibiotic resistance genes and phenotypes [5,16]. Isolates of serotypes 6, 14, 19, and 23 are associated with antibiotic resistance, and they tend to have low immunogenicity[5,7]. They persist in the nasopharynx for a longer period than other serotypes which can play a role in making them more likely to be antibiotic resistant, because of greater opportunity to be exposed to antibacterial drugs [5].

Pili are fibrous appendages expressed on the surface of many bacteria, including *S. pneumoniae*[17]. Pneumococcal pili were described and associated with virulence in 2006[1819]. Two types of pili are encoded by *S. pneumoniae*, PI1 and PI2. Around 30% of all pneumococcal isolates have PI1 present with 50% of those being antibiotic resistant strains. PI1 expression may contribute to fitness advantages such as adherence to lung epithelial cells and nasopharyngeal epithelium[1,17]. While PI1 is well studied, PI2 is less understood; however, it appears that PI2 is also involved with adherence to lung epithelial cells.[1,20].

The 7-valent PCV waslicensed in the US in 2000 and contained serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F[3,21,22]. This vaccine was estimated to provide coverage between 64-69% of the IPD isolates in Brazil[22]. Introduction of PCV7 was projected to result in a 13% reduction of medical costs associated with pneumococcal disease treatment, and this analysis also found that about 3,300 deaths and 270,000 cases of pneumococcal disease could be prevented annually in Brazil[22]. High prices for PCV7 delayed the initial introduction of the vaccine in numerous countries, including Brazil[21].

The 10-valent PCV (PCV10) contains the 7 serotypes in PCV7 as well as serotypes 1, 5, 7F and the 13-valent PCV (PCV13) contains the serotypes in PCV10 plus serotypes, 3, 6A, and 19A[1,3]. Countries in Latin American and the Caribbean introduced PCV10 into their Expanded Program on Immunization (EPI) in 2009[3]. In Brazil, 7-valent PCV has been registered since 2001; however, it was only available to children and adults at high risk for invasive pneumococcal disease in public clinics at no cost[23]. This resulted in a large portion of the Brazilian population not being vaccinated at that time[23]. It was not until 2010, that the 10-valent PCV vaccine was established in Brazil's National Immunization Program at no cost to patients[23]. PCV13 was also introduced in 2010, but it was only available in private vaccination clinics. Due

to PCV13 high cost, only around 8% of children received at least one dose of PCV13[3]. Both PCV10 and PCV13 are still being used in Brazil.

It is estimated that around half a million children under the age of 5 die of pneumococcal disease every year worldwide [24]. In Latin America, pneumonia is a leading cause of death and hospitalization for children under 5 years of age and in adults over 60 years old[4]. There is a need to understand the vaccine impact and epidemiology of *S. pneumoniae* isolates pre and post vaccine introduction periods in Brazil to assess changes in resistance pattern and emergence of non-vaccine serotypes in invasive pneumococcal disease.

The primary objectives of this thesis are: 1) to assess whether there are any changes in serotype prevalence after PCV vaccination in Brazil, 2) to detect and characterize emergence or changes in antibacterial resistance after vaccination, and 3) to examine changes in genotype distribution and presence of pilus genes pre and post vaccination. This study will provide a more detailed understanding of the changes in population structure of *S. pneumoniae* isolates as well as their resistance patterns, potential for increased virulence, and serotype distribution. This information can guide policy makers on the future use of pneumococcal conjugate vaccines and antibacterial treatment options in Brazil.

Chapter 2: Summary of Published Data from Brazil (Literature Review)

The systematic review included searching published studies in PubMed that were specifically conducted in Brazil and that included information on pre-and post- PCV introduction, serotype and genotype changes, resistance, and pilus information. The initial search, with "("Pneumococcal conjugate vaccine" OR "pneumococcal conjugate vaccine" OR "PCV10" OR "PCV13") AND (Brazil OR Brasil)" in the search bar, yielded 82 articles. Upon review a total of 15 articles were chosen because they focused their study on pre-and post-vaccine introduction, as well as contributed information on serotype distribution, genotype changes, and antibacterial resistance. The time range for the studies ranged from 2010-2018. We did not find papers that met the search criteria and included information on pneumococcal pili.

Common methodologies for confirming identification of S. pneumoniae.

Most of the studies followed very similar methodologies for confirming *S. pneumoniae*. For identification Gram stain, colony morphology, optochin susceptibility, bile solubility, and growth on 5% sheep blood agar was used[4,23,25-36]. For serotype, multiplex PCR was used, and for the isolates not able to be resolved with multiplex PCR, the Quellung reaction test was performed. Disk diffusion, E-test, or broth micro-dilution were used to evaluate antibacterial resistance. Sample sizes in the different studies ranged from 82 to 522 isolates[4,23,25-36].

Common data analysis:

The type of analysis varied depending on the specific study. Azevedo et al., de O. Menezes et al., and dos Santos et al. both used Fisher's exact test for analysis[25,27,33]. Neves et al. used a multivariate analysis for the study done in 2017 and for the study conducted in 2018, a chi square test was used[23,29]. Other studies also used chi-square test [4,30,**33**]. Cochran's test and t-test

were other types of analysis used[35,36]. The statistical software program for data analysis also varied by study. Each study used a different statistical program.

Serotype distribution

All 14 articles included in this systematic review researched serotype distribution. In Table 1 information on key details from each study can be found. Out of the 14 articles, two focused solely on serotype distribution in the pre-PCV introduction era: Barroso et al., de O. Menezes et al., and Yoshioka et al, and two articles focused solely on serotype distribution in the post-PCV introduction era: Christophe et al. and Neves et al. (2017). Two studies did not look at serotype distribution by pre- and post- PCV introduction: dos Santos et al. (2015), and Dulius et al. The remaining seven studies included pre- and post PCV introduction periods.

The most common serotypes mentioned in pre PCV introduction periods were: 14, 3, 6B, and 23F. Serotype 14 was the most abundant in all studies, and serotype 3 was found in nine out of the 14 studies. Brandileone et al. found serotype 14 to be the most common in their pre-PCV group[31]. With two post-PCV period (early: 2010-2013; late: 2014-2015), a high number of serotype 14 was still observed in the early post-PCV period, but by the late post-PCV period, it had decreased significantly.

In the post-PCV introduction periods, the most common serotypes found were: 12F, 3, 19A, and 6C[4,23,25-28,30-37]. Serotype 19A and serotype 3 were included in PCV13, but studies have shown increases in these serotypes after vaccine introduction[28]. Brandileone et al, observed relative increases in serotypes 6C, and19A, and they observed an increase in all the age groups for serotype 3[31]. Two other studies also observed slight increases in serotype 12F: Azevedo et al, and dos Santos et al[25,27].

Genotype changes

Five out of 14 studies analyzed genotypic changes pre and post-PCV introduction. Either MLST, PFGE, or MLVA was used to observe genotypic changes[23,25,29,30,32]. Sequence type (ST) 66 is considered one of the most prevalent clone associated with IPD in Salvador, Brazil and other regions of Brazil[25,34]. Azevedo et al found only one isolate belonging to ST66, but for dos Santos et al., ST66 was the most common clonal group [25]. ST156 was another commonly mentioned ST in the studies[30,34,37]. Barroso et al. observed 89% of their 52% penicillin resistant isolates were ST156[30], and Neves et al (2018) also observed ST156 had the highest penicillin resistance in pre- and post-PCV period [37]. Christophe et al. observed two relevant clonal groups to non-PCV10 serotypes: ST320 and ST6403. Also, resistance to antibacterial drugs was evident in isolates belonging to ST320 associated with serotype 19A [32].

Antibacterial Resistance post PCV vaccination

The common methods for evaluating antibacterial resistance included either using the disk diffusion method, broth microdilution, or E-test. Antibacterials evaluated included: penicillin (PEN), erythromycin (ERY), tetracycline (TET), and trimethoprim-sulfamethoxazole (COT). PEN resistance was a focus in many of the studies. Azevedo et al. found no observable differences in antibacterial resistance between pre-vaccine vaccination periods; overall, almost 56% of the isolates were resistant to at least one or more antibiotics[25]. Neves et al (2018) observed an increase of PEN resistance from pre-PCV (24%) to post-PCV introduction (39%) [37]. Barroso et al observed an increase of PEN resistance of 8% in 2000 to almost 20% in 2008[30]. Caierão et al. had the highest percentage of PEN resistant isolates at 42.8%. Almost 80% of isolates that were NS to PEN were also NS to COT and 30% of isolates NS to PEN were also NS to TET in de O. Menezes et al study[26]. Similarly, with Barroso et al, 74% of the isolates NS to PEN were resistant also to COT[30].

Majority of the studies experienced high NS to COT. The percentages of NS to COT ranged from 6%[32]to as high as 62%[37]. Dos Santos et al (2013) observed an increase in susceptibility in the post vaccine introduction group to COT from 31% to 68% for the age group less than 15 years old and from 64-88% in the age group older than 15 years old[27]. In a later study, dos Santos et al (2015), observed COT resistance in 56% of all the isolates collected[34].

Erythromycin is a macrolide commonly used in the empirical treatment of pneumonia according to Brazilian guidelines[32]. Some studies found high susceptibility to ERY: 97%[36]-98%[33,34] , and some studies found no trend in increasing antibacterial susceptibility to erythromycin[25,27]. Neves et al (2017 & 2018), did observe an increase in ERY resistance[23,37]. Only three isolates were resistant to erythromycin pre-vaccination, but almost 28% of the isolates in post-vaccination period were resistant[37], and in their 2017 study, 28% of the isolates were ERY resistant[23]. Christophe et al, also observed ERY resistance with almost 20% of the isolates from the study being NS[32].

Multidrug resistance was address in five studies. Dos Santos et al (2015), found MDR in 4 serotypes: 14, 6B, 23F, and 19F[34]. Similarly, Yoshioka's five MDR isolates were serotype: 14, 6B, and 23F[36], and de O. Menezes's study single MDR isolate was serotype 6B[33]. Neves' studies were the only ones to observe an increase in MDR. In Neves et al 2018, MDR resistance was found among 29.4% of the isolates[37] and in Neves et al 2017, 22% of the isolates were MDR.

Summary of previous studies, limitations and study relevance

Overall, studies looking at serotypes before and after vaccine introduction observed a drop in vaccine-type serotypes such as 14 and 6B, but a high percentage of vaccine-type serotypes after vaccine introduction were still in the population. ST66 and ST156 are common

STs in Brazil. It will be interesting to determine the frequency of these particular STs in this thesis and its association with AMR. A limitation for each of the studies was the focus on defined geographic areas in Brazil, and not on the entire country. Of the 14 selected studies, four focused on the northeast region of Brazil, 6 on the southeast region of Brazil, and three on southern part of Brazil. These are the most populated regions in Brazil which makes sense as to why studies have focused on these areas. The provided dataset has isolates from all 5 major regions in Brazil: North, Northeast, Central-West, Southeast, and South. This thesis therefore focuses on all regions of Brazil, and provides a more detailed analysis of the pneumococcal strain features that is lacking in some of the previous publications. This will allow for a more comprehensive and representative pre- and post-PCV analysis for the entire country. This analysis also includes use of a whole genome sequence approach for characterizing isolates from Brazil which is novel to this study approach. Also, none of the previous studies from Brazil have looked at presence of pilus genes and this analysis will assess changes in the presence of pilus genes and their potential association with serotype and specific clonal complexes.

Chapter 3: Methods

Introduction: Research Questions

The following questions will be answered from the metadata and whole genome sequencing data acquired from samples from Brazil:

1. Is there a significant change in serotype distribution after PCV vaccination in Brazil?
2. Is there an emergence of (or significant changes in) antibacterial resistance after vaccination?
3. Is there a significant difference in genotype distribution and presence of pilus genes pre- and post-vaccination?

Population and Sample: Isolates

Isolates from Brazil were obtained as part of the Global Pneumococcal Sequencing project (www.pneumogen.net). A total of 476 randomly selected invasive isolates were included in this analysis. Isolates were collected from multiple hospitals and laboratories throughout numerous regions in Brazil; 236 of the study isolates were collected in the pre-vaccination period (2008-2009) and 240 were collected in the post-vaccination period (2012-2013). The metadata included information on: location of collection, month and year of collection, age (years and months), gender, clinical manifestation, and specimen source.

Procedures: DNA Extractions and Whole Genome Sequencing

All isolates were cultured on 5% sheep blood agar (SBA), and incubated overnight at 35ºC with 5% $C0_2$. Following incubation, a single colony was picked, then cultured again on 5%

SBA with 5% $C0_2$ with an optochin disk to check for susceptibility. Bile solubility was done when the zone of inhibition was less than 14mm. DNA extractions were done using a modified Qiagen extraction method. Quantifications for the final DNA extractions were determined using Qubit system. DNA extractions were sent to Sanger Institute, UK for whole genome sequencing. Sequencing was performed on Illumina HiSeq or 10X platforms with Truseq chemistry and >=100bp paired-end reads. Sequences were analyzed using the CDC's *Streptococcus* lab pneumococcal typing pipeline to identify serotypes, sequence types (STs), pilus genes, transpeptidase domain amino acid sequences from penicillin-binding proteins (PBPs) 1a, 2b, and 2x, and other resistance features [38,39]. Non-susceptibility to 6 different β-lactams was predicted by assigning a PBP type as previously described [38-40], and correlating this PBP type with phenotypically measured MIC values for isolates with the same type (http://www.cdc.gov/streplab/mic-tables.html), based on current CLSI guidelines [41]. Multidrug resistance was defined as being resistant to ≥3 antibacterial drugs. The eBURST algorithm (eburst.mlst.net) was used to identify groups of related STs (clonal complexes, CC). The PHYLOViZ online application (https://online.phyloviz.net/index) was used to generate minimum-spanning tress.

Statistical Analysis

Statistical analysis was performed on SAS 9.4. Data were divided in to pre- and post-vaccination periods. The pre-vaccination period included isolates collected from January 2008-February 2009, and the PCV period included isolates collected from January 2012- February 2013. For antibacterial resistance, the data were divided between CSF isolates non-CSF isolates due to different MIC cut-offs. Along with chi-square analysis, contingency tables on OpenEpi were also used to determine any statistically significant associations (alpha=0.05).

Ethical Considerations:

This analysis was determined to be IRB-exempt because it is an analysis of secondary data and all data were de-identified prior to analysis. Prior to data collection, all portions of the study were reviewed by Emory University's Institutional Review Board (IRB00103706) and determined to meet the criteria for exemption.

Chapter 4: Results and Discussion

Demographics:

General characteristics and demographics of the 476 study isolates are summarized in Table 2 and Table 3 for pre-PCV introduction and post-PCV introduction, respectively. Overall, 318 (66.8%) isolates were from ≤5 years old, and 158 (33.2%) isolates were > 5 years old. There were more males in both periods 137 (58.1%) males in pre-PCV and 146 (60.8%) in post-PCV. Pneumococci evaluated in this analysis were isolated from blood 240 (50.4%), CSF 210 (44.1%), pleural fluid 23 (4.8%), and sterile fluid 3 (0.6%). The reported associated clinical manifestations were meningitis (237, 49.8%), bacteremia (135, 28.4%), pneumonia (102, 21.4%), and abscess (2, 0.4%). There was a statistically significant difference from pre-PCV introduction to post-PCV introduction with bacteremia increasing from 19.1% to 37.5% (p-value <0.0001) and meningitis decreasing from 59.3% to 40.4%. (p-value <0.0001).

Serotypes:

A total of 47 different serotypes were identified; one isolate was non-typeable. Overall, the most prevalent serotypes pre-PCV introduction were 14 (29.7%), 6B (11.9%), 3 (7.6%), 19A (6.4%), and 23F (5.9%) (Figure 1). Three of those serotypes (14, 23F, and 6B) were included in PCV7 and PCV10, and two additional serotypes (3, and 19A) were included in PCV13. Post-PCV introduction, the most prevalent serotypes were 14 (9.2%), 19A (8.8%), 3 (8.8%), 12F (4.6%), and 6A (4.6%). The odds IPD was due to serotype 14, serotype 6B, or serotype 23F before PCV introduction than the odds of IPD due to a non- serotype 14, 6B, or 23F after PCV introduction was: 4.1 (p-value <0.0001), 3.4 (p-value <0.0009), and 2.5 (p-value=0.07), respectively. Serotype 3,6A, and 19A are included in PCV13, and experienced anincrease from the pre- to the post-PCV period: 14.7%, (OR=0.9, p-value= 0.7), 35.1% (OR=0.7, p-value=0.5), and 37.6% (OR=0.7, p-value=0.3), respectively.

In ≤5 years old age group, the difference in serotypes pre-and-post-PCV introduction can be seen in Figure 2. The most prevalent serotypes for this age group pre-PCV introduction were: 14 (38.6%), 6B (16.5%), 19A (7.6%), 18B:18C (5.7%), and 23F (5.1%), and post-PCV: 19A (12.5%), 14 (10.6%), 3 (8.8%), 6A (5.6%), and 6C (5.6%). The odds that IPD was due to serotype 19A or serotype 3 in ≤5 years old age group in post-PCV introduction than the odds that IPD was due to a non-serotype 19A or 3 pre-PCV introduction was 0.6 (p-value: 0.15) and 0.3 (p-value= 0.04), respectively. The odds that IPD due to serotype 14 or 6B in ≤5 years old age group in pre-PCV introduction than the odds of IPD due to non-serotype 14 post-PCV introduction was 5.6 or 4.3, respectively. Serotype 6C went from not being present in the pre-PCV period to being associated with >5% (p-value=0.002) IPD during the post- PCV introduction period.

In the over 5 year old age group, the difference in serotypes pre-and-post-PCV can be seen in Figure 3 Pre-PCV, the most common serotypes were 3 (16.7%), 14 (11.5%), 23F (7.7%), 4 (6.4%), 7F (6.4%), and post-PCV, the most common were 3 (8.8%), 8 (7.5%), 9N (7.5%), 12F (6.3%), 14 (6.3%). The odds IPD was due to serotype 3 in >5 year old age group before PCV introduction was 2.1 (p-value <0.0001) times higher than the odds of IPD due to a non- serotype 3 after PCV introduction. There was a decrease in serotype 23F (83.7%, p-value=0.01). There was a decrease in serotype 23F (6.4%, p-value=0.05).

Genotype Distribution and Pilus Genes

Overall, 110 different STs were observed and clustered in 30 clonal complexes (CCs) (Table 4). The most prevalent ST was ST156, both pre- (50, 21.4%) and-post-PCV (23, 10.4%) introduction; of the 73 ST156 isolates, 68 were serotype 14 and five were serotype 9V. ST66 was another common ST in both pre-and-post-PCV with a total of 21 isolates; 11 of the isolates

were serotype 9N, nine were serotype 14, and one was serotype 19F. An eBURST diagram of all pre-PCV STs (Figure 4) showed that the majority of isolates belonged to ST156, ST66, ST338, ST180, and ST193, and a diagram of post-PCV STs (Figure 5) showed that the majority of the isolates belonged to ST156, ST66, ST53, ST320, and ST180. A minimum spanning tree was generated to compare ST distribution pre-and-post-PCV introduction (Figure 6), and for the relationship between ST and serotype (Figure 7).

In the pre-PCV introduction group, sequences coding for presence of PI1 or PI2 were detected in 43.2%; 83 (35.2%) were PI1, 14 (5.9%) were PI2, and 5 (2.1%) were positive for both PI1 and PI2. In the post-PVC group, there were only 50 isolates (20.8%) out of 240 isolates that were positive for PI1 or I-2: 30 (12.5%) were positive for PI1, 8 (3.3%) were PI2, 12 (5.0%) were both PI1 and PI2.

There was a strong correlation between the presence of pilus genes and specific clonal complexes (Figure 8), with the majority of isolates with pili belonging to either CC156 or CC236 (Table 5). PI1 had the most diverse serotype distribution with 9 different serotypes that PI1 coding sequences. For the isolates in CC156, a large quantity were serotype 14 (96.7% pre-PCV, and 95.8% post-PCV), and all of them were positive for only PI1. CC236 were all positive for both PI1 and PI2, and were either serotype 19A or 19F. Isolates positive for PI2 belonged to ST191 (9 pre-PCV, 7 post-PCV), ST304 (5 pre-PCV only), or ST615 (1 post-PCV only), and were either serotype 7F or 1 (Figure 9). Minimum spanning trees were generated to illustrate the relationship between the presence of pilus genes and ST (Figure 8).

Antibacterial Resistance and Multidrug Resistance

Overall, 81.3% of the isolates were predicted to be non-susceptible (NS) to at least 1 antibacterial (77.1% pre-PCV, and 85.4% post-PCV); 78.6% of all isolates were resistant to

cotrimoxazole, and 39.7% were non-susceptible to penicillin. Prior to vaccine introduction, isolates resistant to at least one class of antibacterials belonged to serotypes: 14 (38.5%), 6B (15.4%), and 23F (8.8%); post-PCV, they belonged to serotypes: 14 (10.7%), 19A (10.7%), and 3 (9.3%). The proportion of NS isolates belonging to serotype 14 or 6B decreased significantly after vaccine introduction: by 27.7% (p-value <0.001) and 11.0% (p-value <0.001), respectively. On the other hand, the proportion of serotype 3 among NS isolates increased significantly by 8.2% (p-value <0.001); most of the NS serotype 3 isolates (14/21) belonged to ST180. Among the 189 penicillin non-susceptible (PNS) isolates, the most common genetic lineage was CC156/Serotype 14/9V (n=82, 43.4%), followed by the CC338/Serotype 23F lineage (n=15, 7.9%), and the CC118/Serotype 19A lineage (n=13, 6.9%); the only significant change after vaccine introduction was observed in the CC156 lineage, which represented 76.6% of PNS isolates pre-PCV, but decreased to 14.6% (p<0.01) post-PCV.

The proportion of isolates predicted to be non-susceptible to penicillin, meropenem, and ceftriaxone decreased significantly after PCV introduction (Table 6). The relative risk that an isolate was NS to penicillin decreased by -29 fold in the post-PCV introduction period when compared to the pre-PCV period. Meropenem NS and ceftriaxone NS also decreased by -31 fold and -32 fold, respectively. Cotrimoxazole was the only antibiotic to increase in NS by 18 fold in the post-PCV introduction period when compared to the pre-PCV period.

Among 210 non-CSF isolates, the proportion of isolates that were non-susceptible to ≥1 antibacterial drug was 75.4% in the pre-PCV period and 92.9% during the PCV period (RR= 0.82; p=0.001) (Table 7). As was the case among CSF isolates, the relative risk that an isolate was NS to meropenem decreased by -31 fold in the post-PCV introduction period when compared to the pre-PCV period. Cefuroxime also decreased by -25 fold from pre to post-PCV

introduction periods. Contrary to what was observed among CSF isolates, there was a statistically significant increase in NS against five of the ten antibiotics examined. The largest increase was against tetracycline which showed an increase of 15.2% (p-value=0.0035) from 13.6% to 28.85%. Erythromycin increased 8.5% (0.0438), and penicillin, amoxicillin, and ceftriaxone all increased 5.5% (p-value=0.0265).

Multidrug resistance (MDR) was observed among both CSF and non-CSF isolates, and both groups showed an increase in the proportion of MDR from the pre-PCV to the post-PCV period (12.4% difference; (p-value=0.08)). Among CSF isolates, there was an increase of 3.2% (p-value=0.49) and among non-CSF isolates, there was an increase of 7.2% (p-value=0.08), in MDR after vaccine introduction. The most common genetic lineage among MDR isolates was CC320/Serotype 19A (n=13, 21.7%); most of these isolates were isolated during the post-PCV period.

Discussion:

The main objective of this thesis was to evaluate changes in serotype, genotype, and antibacterial resistance after PCV introduction in Brazil, where few studies have focused on the molecular epidemiology of the IPD population. Examining the evolution of *S. pneumoniae* at the molecular level has provided more information that could potentially help guide new policies for the future use of PCVs in Brazil.

Prior to the introduction of PCV, the most common serotypes in Brazil were serotypes 14, 6B, 6A, 18C, and 19F ; for this study, the two dominant serotypes were 14 (29.66%), and 6B (11.86%), supporting the previous data[42]. While serotype 3 was not as common in McIntosh et al., it was a very dominant serotype in the studies conducted by Leite et al, Azevedo et al, and Caerãio et al[23,25,26,42]. It was also somewhat common in this study with 7.63% of the study isolates being serotype 3. In previous studies, a reduction of vaccine-type serotypes in association with an increase in non-vaccine serotypes was observed after the introduction of PCV. While we observed a significant drop of 37.31% (p-value=<0.001) of isolates with PCV10 serotypes, the most common serotypes after vaccine introduction still included vaccine types 14. Contrary to what was observed by dos Santos et al., there was an increase in serotype 19A from pre-PCV to post-PCV among our isolates, and similarly to what was reported by Azevedo et al., a small increase of 12F was also observed post-PCV in this study[25,27].

High proportions of resistant isolates have been observed among some vaccine types. There was a significant drop of penicillin NS from the pre-PCV to the post-PCV period among CSF isolates, and this was associated with serotype 14. However, for non-CSF isolates an increase in penicillin NS was observed.. Even with high vaccine coverage in Brazil, other studies have shown penicillin non-susceptibility was still high in children under the age of 6 post-PCV

introduction[27]. Contrary to dos Santos et al., who observed an increase in susceptibility of COT, we found an increase of NS among CSF isolates[27]. This is similar to other Brazilian studies that have reported high resistance rates to COT, and may be due to the popularity of this drug that requires no medical prescription[33]**.** According to Neves et al., the emergence of MDR serotype 6C has been a growing concern in Brazil[29]. We observed a significant increase of serotype 6C among ≤5 years old post-PCV introduction (p-value=0.002), as well as a significant association of serotype 6C with MDR (p-value= 0.0003).

According to dos Santos et al (2015), ST66 has been persistently dominant over 17 years of surveillance in Brazil, and is an important factor for maintaining the penicillin resistance rate[34].

ST156 and ST180 were the most common ST reported by Caierão et al[26]. Like Caierão et al., ST156 and ST180 were common in this study for pre-PCV, but for post-PCV, ST156 and ST66 were the most common. Christophe et al. observed an increase in non-PCV10 serotype 19A related to ST320 that were resistant[32]. ST320 was also common post PCV introduction, and 33% of those isolates were serotype 19A. In this study, all ST320 19A isolates were also MDR. A study looking at carriage isolates, Neves et al., observed ST724 and ST386 to be the most common[29]. Serotype switching was observed in Neves et al. for ST338 with 23F in pre-PCV to serotypes 23A, 23B and 23F in post-PCV introduction[29]. This was not the case in this study. In the pre-PCV period, all ST338 were serotype 23F, and in post-PCV introduction, all ST338 except one were 23F, the other was 15B.

No similar study involving pilus genes has been conducted in Brazil; however, [1]a study conducted in Peru pre and post introduction of PCV7[1], examined the relationship between the presence of pilus genes and serotype. Similarly to what was observed by Hawkins et al., we

found PI-1 to be most commonly associated with serotype 14 both pre and post vaccine introduction. Among our isolates, PI-2 was associated with serotypes 1 and 7F, while in Peru, PI-2 was exclusively found in association with serotype 1. The presence of both pilus genes (PI-1 + PI-2) was associated with both 19A and 19F pre-PCV10, but only with 19A after vaccine introduction, most likely because serotype 19F was not observed among post-PCV isolates. We also found a strong correlation with the presence of pili and CCs just like Hawkins et al. CC156 was a majority of the isolates positive for the presence of pili and it was also the majority in our study. Out of the 152 isolates that were positive for the presence of pili, 138 (90.8%) were resistant to at least one antibiotic. There was an association with the presence of pilus genes and resistance (p-value= 0.03), and there was also an association with the presence of PI-1 and resistance (p-value <0.0001).

Strengths and Weaknesses:

Strengths:

The availability of invasive pneumococcal isolates allowed for a comprehensive analysis of molecular factors associated with invasive pneumococcal disease pre- and post-vaccine introduction in Brazil. This study used a whole genome sequencing approach to identify important pneumococcal strain features such as presence of pilus genes that allowed for its association with clonal complexes and serotypes that were not previously available on isolates from Brazil.

Weaknesses:

Some limitations include a small sample size and limited data collection period after PCV10, since isolates were collected only 2 years after the implementation of PCV10 in Brazil's National Immunization Program. While some significant changes were observed, the full impact

of the vaccine may not have been fully represented. Additional analyses over longer periods of time would be helpful to further assess the continued impact of vaccine introduction. Another limitation from the sampling was stratifying the isolates by age and pre-and-post-PCV groups. This resulted in small sample sizes, especially for the over 5 year old category, and that could affect statistical power.

Implications:

With almost half a million children under the age of 5 dying of pneumococcal disease every year around the world and with pneumonia leading the cause of deaths and hospitalization in children under 5 in Latin America, prevention and treatment interventions are crucial for improving public health not only in Brazil but other developing countries[4,23,24]. Ongoing surveillance in Brazil will allow monitoring for continued changes in the *S. pneumoniae* population, and measure vaccine impacts on serotype prevalence and antibacterial resistance. Understanding these changes could help guide the development of future vaccines and prevention efforts in Brazil.

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Tables

Table 1: Summary of key details from systematic review

Author	Year	Region*	City	Study Population	Sample Size	Source	Years collected	Vaccine Focus
Azevedo et al.	2016	NE	Salvador	All ages	148 cases	CSF	2008-2012	PCV10
Barroso et al.	2010	SE	Rio de Janeiro	All ages	1,272 cases	Blood, CSF	2000-2008	PCV10
Brandileone et al.	2018	ALL	ALL	All ages	10,305 isolates	Blood, CSF, other sterile sites	2005-2015	PCV10
Caierão et al.	2014	S	Porto Alegre	All ages	325 isolates	Blood, CSF, pleural fluid, ascites, peritoneal fluid, joint fluid	2007-2012	PCV10
Christophe et al.	2018	S	Porto Alegre	Adults over 50 years old	102 isolates	Blood, CSF, pleural fluid, ascites, pericadial and joint fluid	2013-2015	PCV10
de O. Menez et al.	2011	NE	Salvador	All ages	421 cases	CSF	2000-2007	PCV7, PCV10
dos Santos et al.	2013	SE	Butantã	All ages	259 isolates	Blood, CSF, pleural fluid	2006- 2012	PCV10
dos Santos et al.	2015	NE	Salvador	All ages	854 cases	CSF	1996-2012	PCV10
Dullius et al.	2018	S	Porto Alegre	Adults	118 isolates	Blood, CSF, abdominal fluid	2005-2016	PCV10, PPV23
Leite et al	2015	NE	Salvador	All ages	82 patients	Blood, CSF	2010-2013	PCV10
Medeiros et al	2017	SE	Sâo Paulo	All ages	796 cases	Blood, CSF, other sterile sites	1998-2017	PCV10
Neves et al.	2017	SE	Niterói	Children under 6 years old	522 cases	NP swabs	2014	PCV10
Neves et al.	2018	SE	Niterói	Children under 6 years old	256 isolates	NP swabs	2009-2010, 2014	PCV10, PCV13
Yoshioka et al	2010	SE	Sâo Paulo	Children under 6 years old	107 children	Blood, pleural fluid	2003-2008	PCV10, PCV13

*NE: Northeast, SE: Southeast, S: South

Table 2: Demographics of Pre-PCV isolates

	Pre-Vaccination	
Variable	*Count*	*Percent*
Age		
≤5 years old	158	66.95
>5 years old	78	33.05
Gender		
Female	97	41.45
Male	137	58.55
Source		
Blood	102	43.22
CSF	126	53.39
Pleural Fluid	7	2.97
Sterile Fluid	1	0.42
Clinical Manifestation		
Abscess	0	0
Bacteraemia	45	19.07
Meningitis	140	59.32
Pneumonia	51	21.61

Table 3: Demographics of Post-PCV isolates

	Post-Vaccination	
Variable	*Count*	*Percent*
Age		
≤5 years old	160	66.67
>5 years old	80	33.33
Gender		
Female	91	38.4
Male	146	61.6
Source		
Blood	138	57.5
CSF	84	35
Pleural Fluid	16	6.67
Sterile Fluid	2	0.83
Clinical Manifestation		
Abscess	2	0.83
Bacteraemia	90	37.5
Meningitis	97	40.42
Pneumonia	51	21.25

Table 4: Identified Clonal Complex with associated serotypes

Clonal Complex	ST	n	Pre	Post	Serotypes
CC156	156	73	50	23	14 (68), 9V (5)
	12506	2	2	0	14 (2)
	162	3	2	1	9V (3)
	2335	1	1	0	14 (1)
	12507	1	1	0	14 (1)
	1556	1	1	0	14 (1)
	12508	1	1	0	14 (1)
	12505	1	1	0	14 (1)
	12839	1	1	0	14 (1)
CC66	66	21	9	12	14 (9), 9N (11), 19F (1)
	67	1	1	0	15A (1)
	737	3	1	2	7C (3)
	12837	1	1	0	14 (1)
	12487	2	2	0	14 (2)
	73	3	1	2	15A (3)
	11327	4	1	3	13 (4)
	2216	1	0	1	15A (1)
CC1118	1118	4	2	2	19A (4)
	2878	3	2	1	19A (3)
	2880	2	1	1	19A (2)
	9838	1	0	1	19A (1)
	53	8	1	7	8 (8)
	12574	1	0	1	8 (1)
	62	6	1	5	11A (6)
CC177	177	2	2	0	19F (2)
	12513	1	1	0	19F (1)
	3013	1	1	0	19F (1)
	12512	1	1	0	19F (1)
	12510	1	1	0	19F (1)
CC193	193	12	11	1	18B:18C (8), 18B (1), 18C (2)
	1358	1	1	0	18B:18C (1)
	12838	1	1	0	18B:18C (1)
	12517	1	1	0	18B:18C (1)
	1228	1	0	1	15A (1)
	2814	1	0	1	18B (1)
	193*	1	0	1	11A (1)
CC338	338	14	8	6	23F (13), 15B (1)
	12835	1	1	0	23F (1)

	3163	1	1	0	23F (1)
	2777	3	0	3	6C (3)
	338*	1	0	1	23A (1)
CC7027	7027	2	1	1	16F (2)
	733	3	3	0	19A (3)
	12836	1	1	0	16F (1)
	2258	2	2	0	16F (2)
	2258*	1	0	1	16F (1)
CC90	90	6	6	0	6B (6)
	12495	1	1	0	6B (1)
CC315	315	4	3	1	6B (4)
	11315	1	1	0	6B (1)
	386	7	2	5	6B (2), 6C (5)
CC724	724	7	3	4	6B (6), 6A (1)
	12465	1	1	0	6B (1)
	5847	2	2	0	6B (1), 6A (1)
	724*	1	0	1	6B (1)
CC236	236	2	2	0	19F (2)
	320	12	1	11	19A (12)
	202	1	1	0	19A (1)
	11326	1	1	0	19F (1)
	1451	1	0	1	19A (1)
CC751	751	3	2	1	6B (3)
	12514	1	1	0	6B (1)
	750	1	1	0	6B (1)
	12841	1	1	0	6B (1)
	11787	1	0	1	6B (1)
CC8640	8640	2	1	1	19A (2)
	9793	1	1	0	19A (1)
CC6146	6146	1	1	0	14 (1)
	15	2	1	1	14 (1)
	7438	1	0	1	16F (1)
CC945	945	1	1	0	23B (1)
	727	1	1	0	23B (1)
	42	2	0	2	23A (2)
	42*	1	0	1	23A (1)
CC12840	12840	1	1	0	24F (1)
	72	3	1	2	24F (1), 24A/B/F (2)
CC241	241	5	1	4	18A (5)
	5063	1	1	0	18A (1)
CC6403	6403	7	1	6	22F (7)

	698*	1	0	1	35C/42 (1)
	214	1	0	1	22F (1)
CC458	458	7	1	6	3 (7)
	11332	2	0	2	3 (2)
CC230	230	1	0	1	24F (1)
	276	2	0	2	19A (2)
	230*	1	0	1	24B (1)
	2307	1	0	1	19F (1)
CC42	42	2	0	2	23A (2)
	42*	1	0	1	23A (1)
	945	1	0	1	23B (1)
	727	1	0	1	23B (1)
CC770	770	4	0	4	4 (3), NT (1)
	4269	1	0	1	6A (1)
CC393	393	4	0	4	25A (2), 38 (2)
	755	2	1	1	38 (2)
CC4913	4913	3	0	3	15A (3)
	4913*	1	0	1	15A (1)
CC473	473	2	0	2	6A (2)
	1876	2	0	2	6A (2)
CC742	742	3	0	3	10A (3)
	742*	1	0	1	10A (1)
CC766	766	2	1	1	15B (2)
	766*	1	0	1	15C (1)
	766**	1	0	1	15B (1)
CC4877	4877*	1	0	1	35B (1)
	4877	1	0	1	35B (1)
CC7438	7438	1	0	1	16F (1)
	15	1	0	1	14 (1)
CC4978	4978	3	2	1	6B (2)
	4977	1	0	1	6B (1)
Total		**337**	**165**	**172**	

* single locus variant

** double locus variant

Table 5: Association between Presence of Pilus genes and Clonal Complex/Sequence Type

	Pre-PCV					Post-PCV				
CC/ST	**PI1**	**PI2**	**PI1 + PI2**	**% of ST**	**p-value**	**PI1**	**PI2**	**PI1 + PI2**	**% of ST**	**p-value**
CC156	58			96.7	<0.001	23			95.8	<0.001
CC177	5			83.3	0.04452					
CC90	7			100.0	<0.001					
CC315	4			66.7	0.3259					
CC236			5	100.0	<0.001			12	100.0	<0.001
CC8640	2			100.0	<0.001	1			100.0	<0.001
CC766	1			100.0	<0.001	3			100.0	<0.001
CC4877						1			50.0	0.3097
ST191		9		100.0	<0.001		7		100.0	<0.001
ST304		5		100.0	<0.001					
ST615							1		100.0	<0.001
ST497	1			100.0	<0.001					
ST735	1			100.0	<0.001					
ST7206	2			100.0	<0.001					
ST11305	1			100.0	<0.001					

Table 6: Antibiotic non-susceptible CSF Isolates Pre-and Post-PCV Introduction

Antibiotic	% NS Pre	%NS Post	MIC90	p-value
PEN	37.30	23.81	2.0	0.0399*
AMO	0.89	2.38	2.0	0.3423
MER	21.43	10.71	0.5	0.0436*
TAX	19.05	9.52	1.0	0.0599
CFT	21.43	8.52	1.0	0.0233*
CFX	22.22	14.29	>2	0.1513
ERY	11.11	14.29		0.4938
CLI	9.52	10.71		0.7782
COT	70.63	86.90		0.0059*
TET	17.46	27.38		0.0861
MDR	11.11	14.29		0.4938

NS = non-susceptible; PEN= Penicillin; AMO= Amoxicillin; MER= Meropenem; TAX= Cefotaxime; CFT= Ceftriaxone; CFX= Cefuroxime; ERY=Erythromycin; CLI= Clindamycin; COT= Cotrimoxazole; TET= Tetracyline; MDR= Multidrug Resistance

*p-value ≤ 0.05
significant

Table 7: Antibiotic non-susceptible non-CSF Isolates Pre-and Post-PCV Introduction

Antibiotic	% NS Pre	%NS Post	MIC90	p-value
PEN	0.91	6.41	2.0	0.0265*
AMO	0.91	6.41	2.0	0.0265*
MER	37.27	17.95	0.5	0.0004*
TAX	0.91	0.00	1.0	0.2328
CFT	0.91	6.41	1.0	0.0265*
CFX	39.09	21.79	2.0	0.0022*
ERY	8.18	16.67		0.0438*
CLI	7.27	14.10		0.0829
COT	80.91	78.85		0.6801
TET	13.64	28.85		0.0035*
MDR	8.18	15.38		0.0793

NS = non-susceptible; PEN= Penicillin; AMO= Amoxicillin; MER= Meropenem; TAX= Cefotaxime; CFT= Ceftriaxone; CFX= Cefuroxime; ERY=Erythromycin; CLI= Clindamycin; COT= Cotrimoxazole; TET= Tetracyline; MDR= Multidrug Resistance

p-value ≤ 0.05
significant

Figures

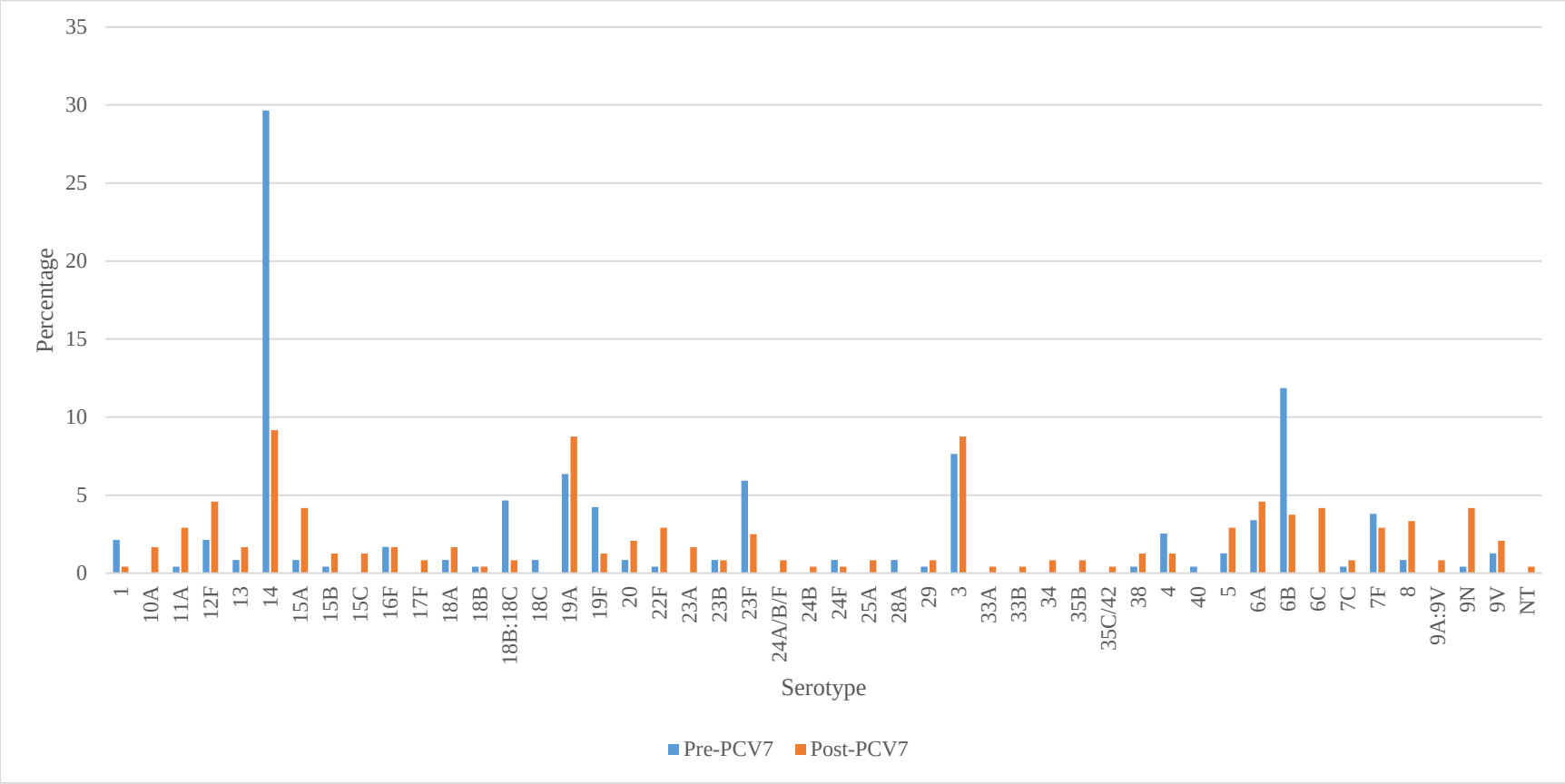

Figure 1: Distribution of Serotype Pre-and Post-PCV

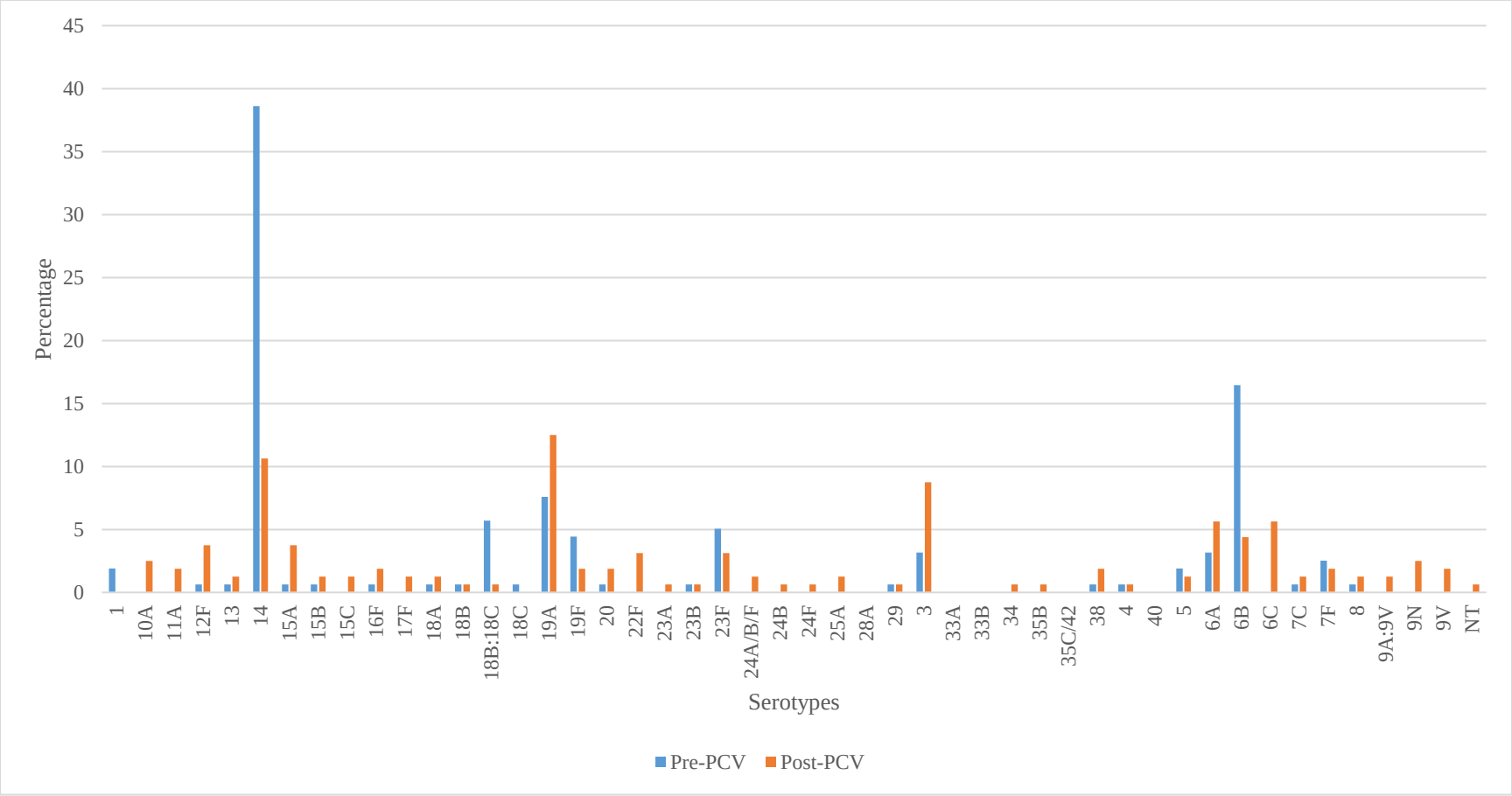

Figure 2: Distribution of Serotypes for Age ≤ 5 Years Old Pre- and-Post-PCV

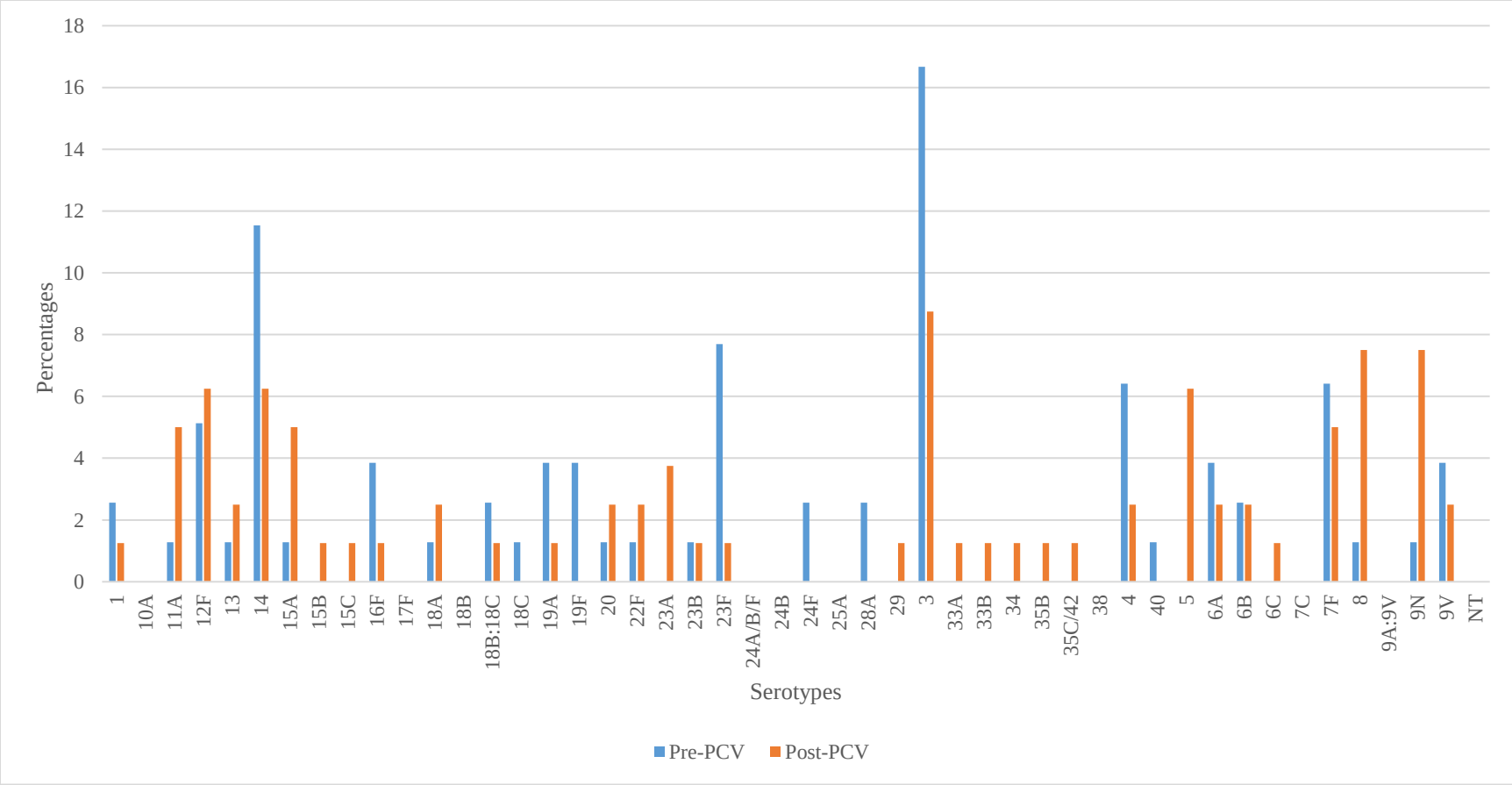

Figure 3: Distribution of Serotypes for Ages > 5 Years Old Pre-and-Post-PCV

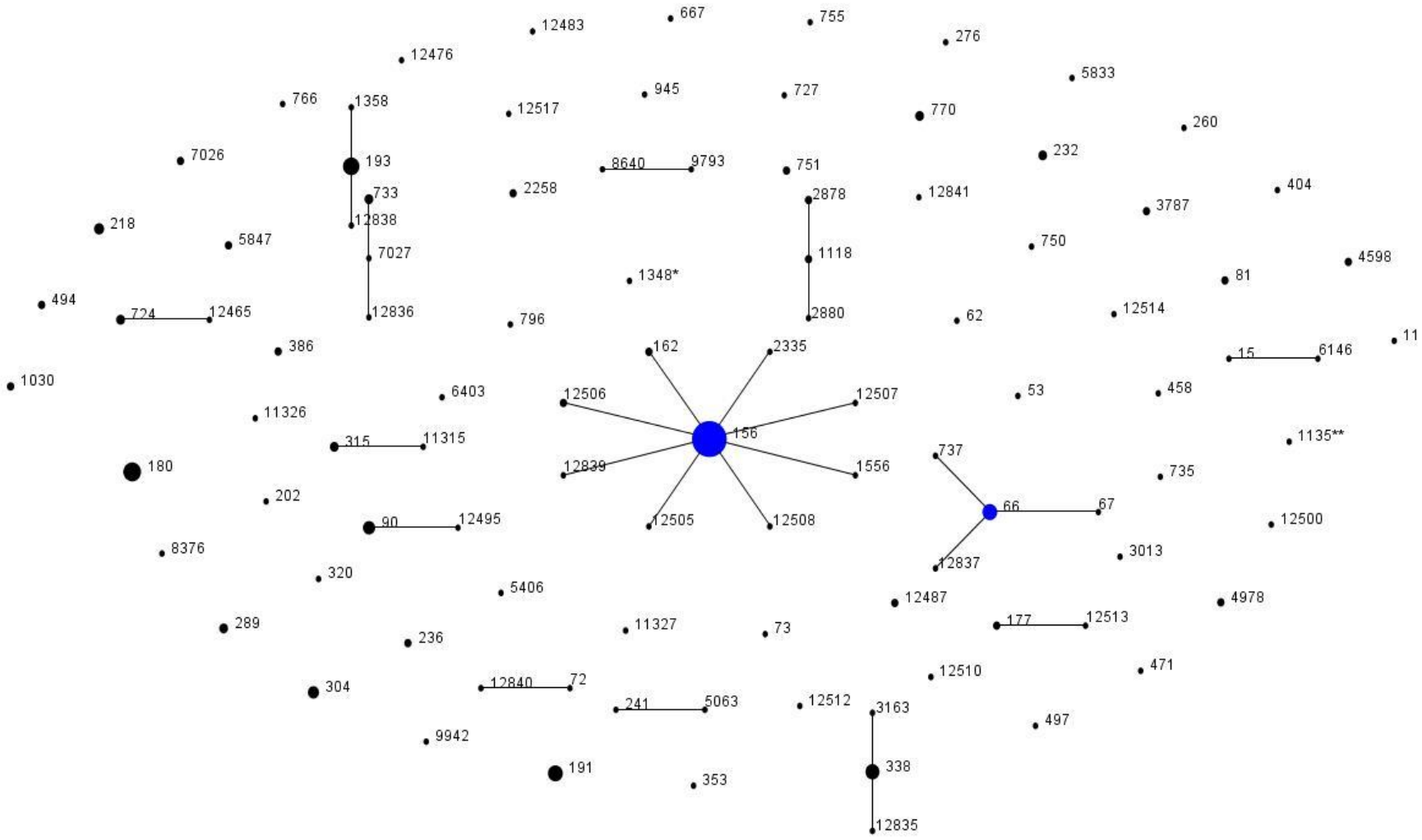

Figure 4: Clonal complex diagram of pre-PCV isolates

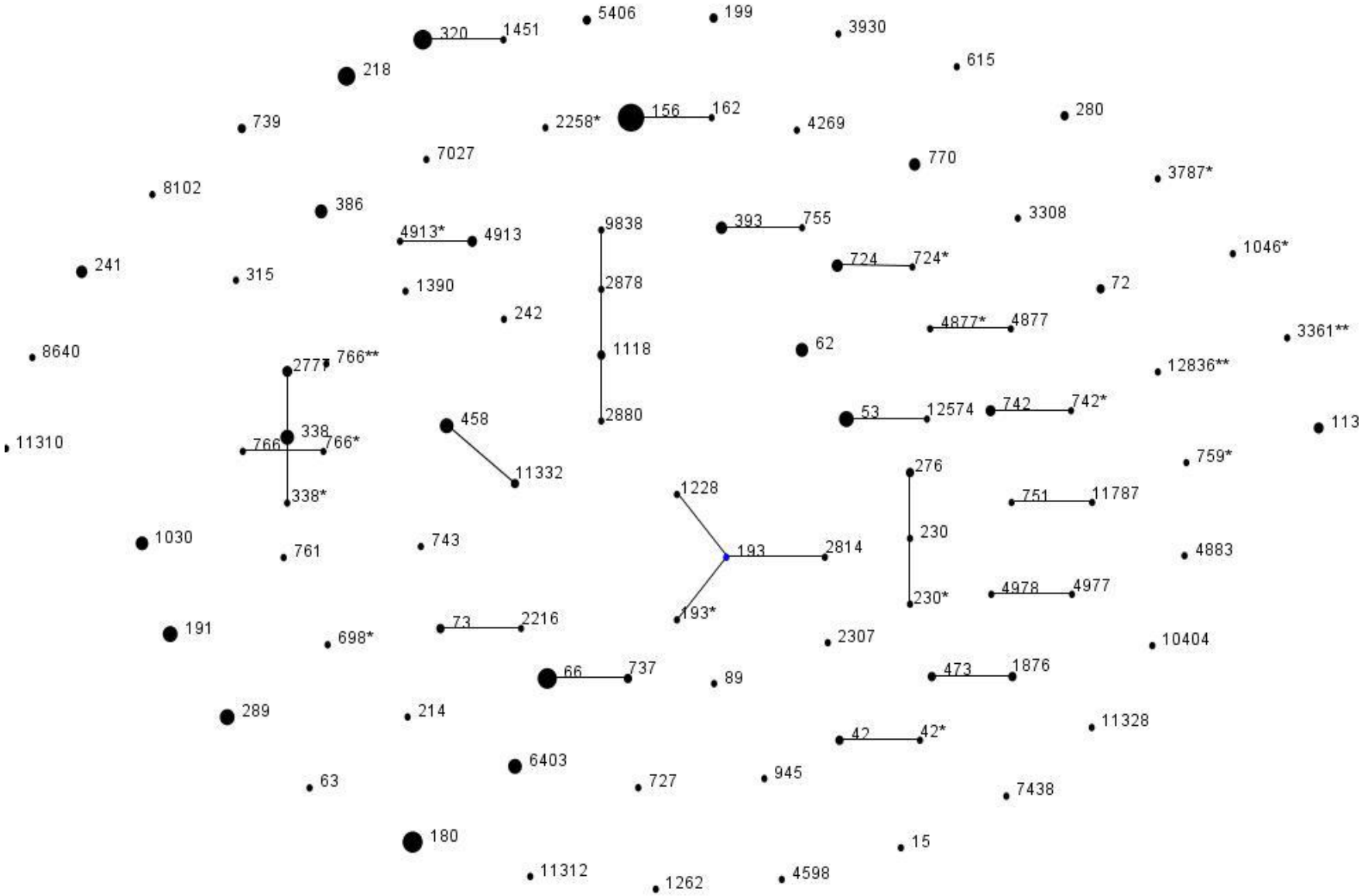

Figure 5: Clonal complex diagram of post-PCV isolates

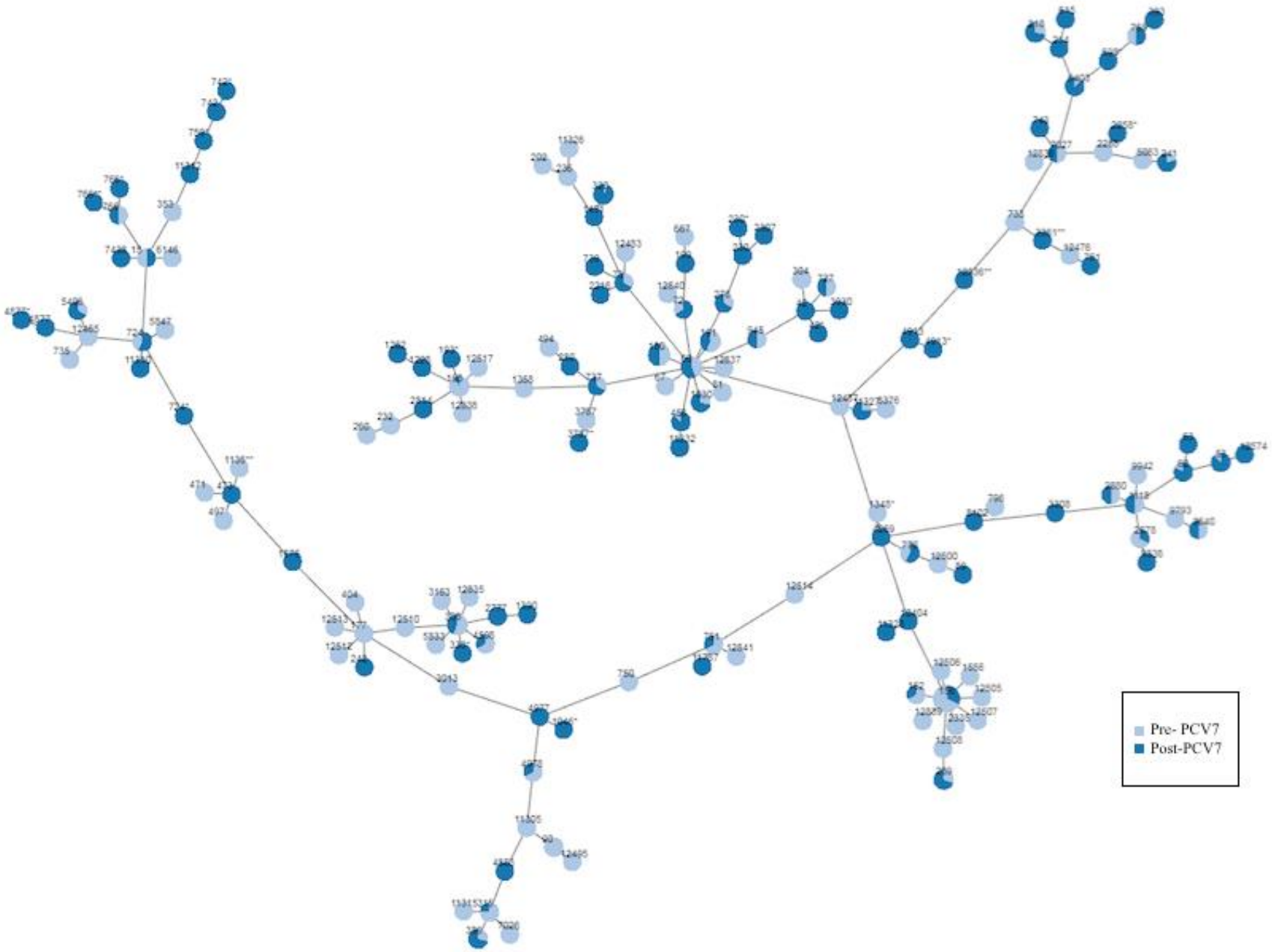

Figure 6: Minimum Spanning Tree of Sequence Type distribution Pre-and-Post-PCV

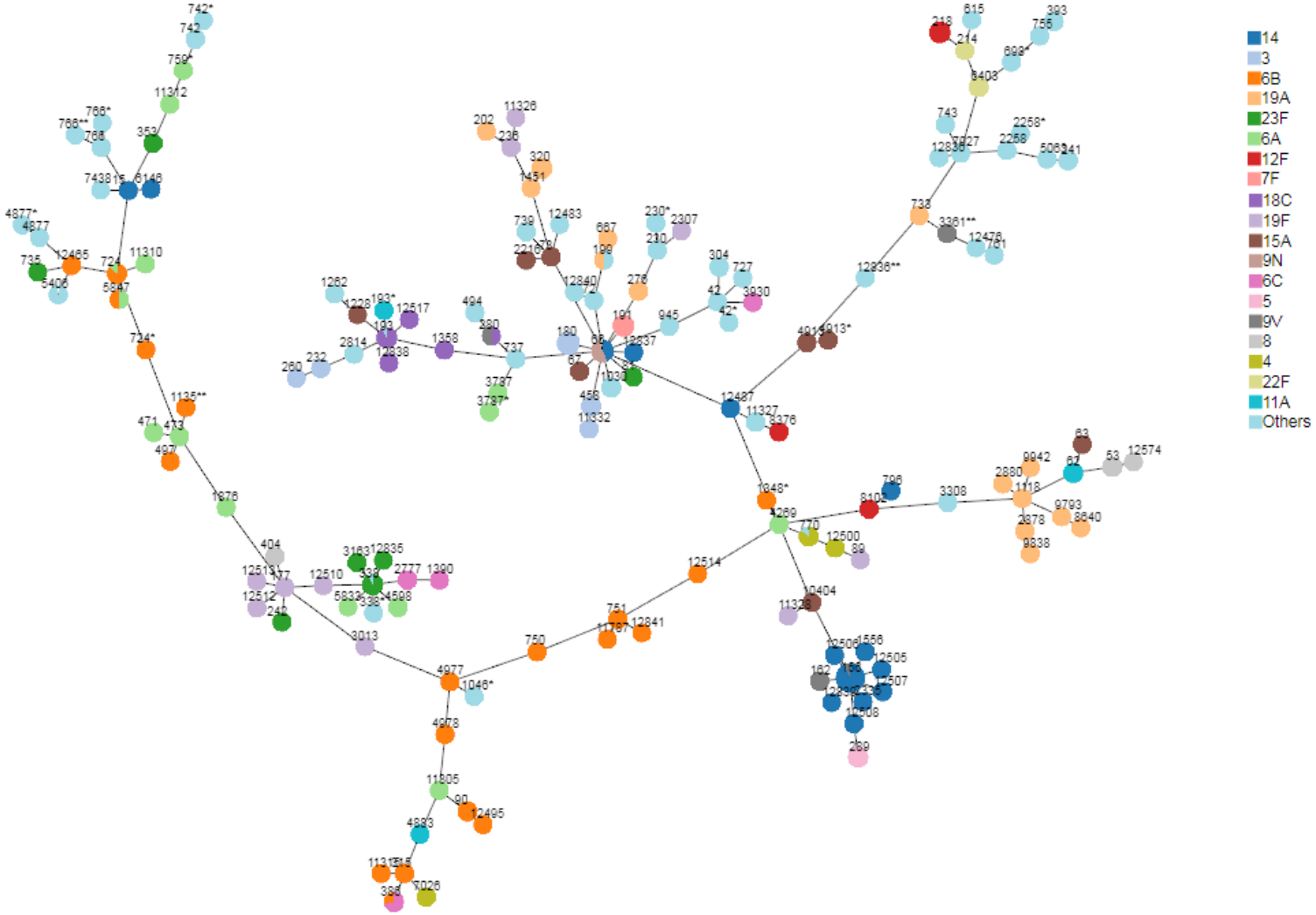

Figure 7: Minimum Spanning Tree showing the relationship between ST and Serotype.

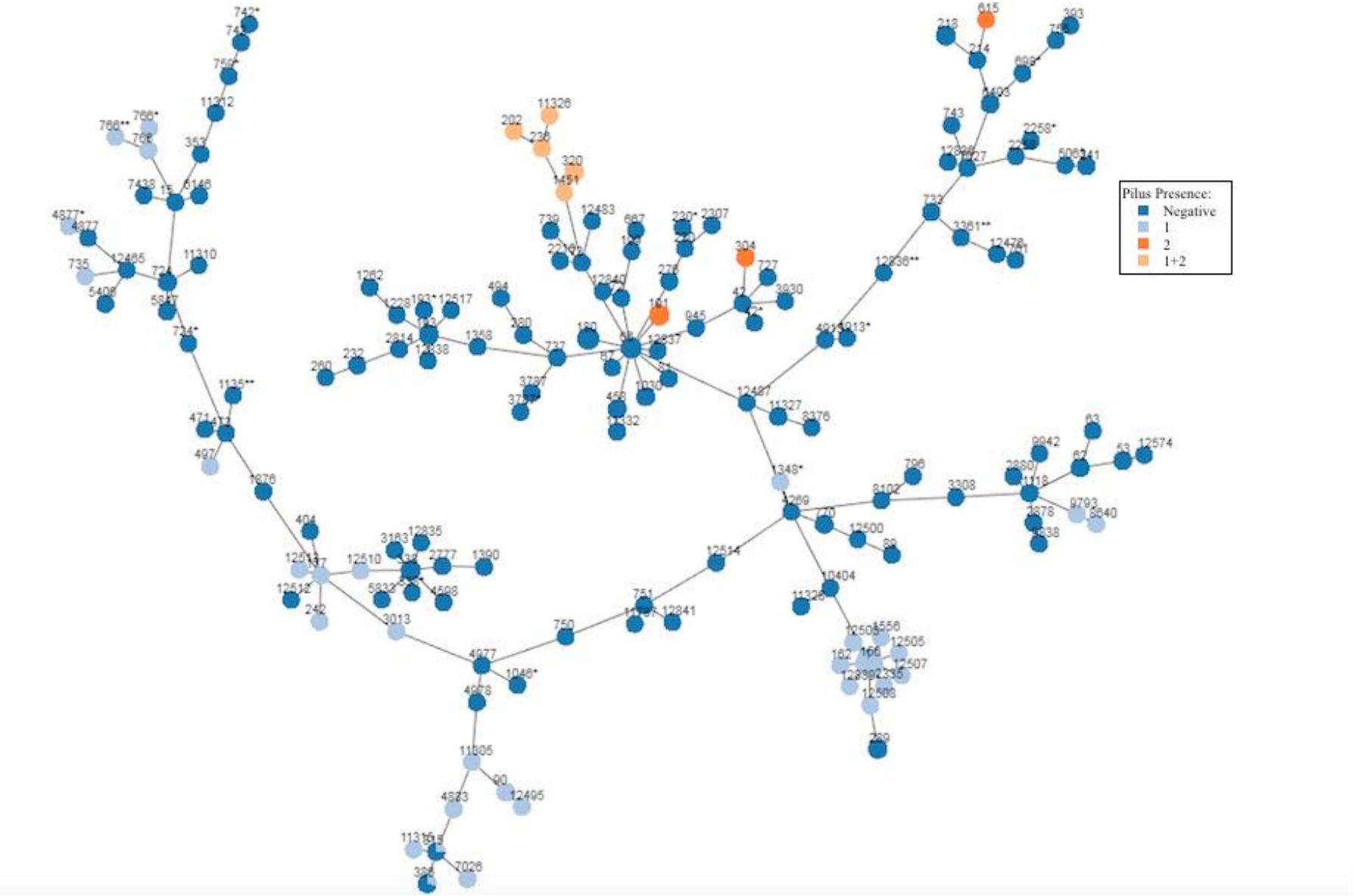

Figure 8: Minimum Spanning Tree showing the relationship between ST and for Pilus Presence

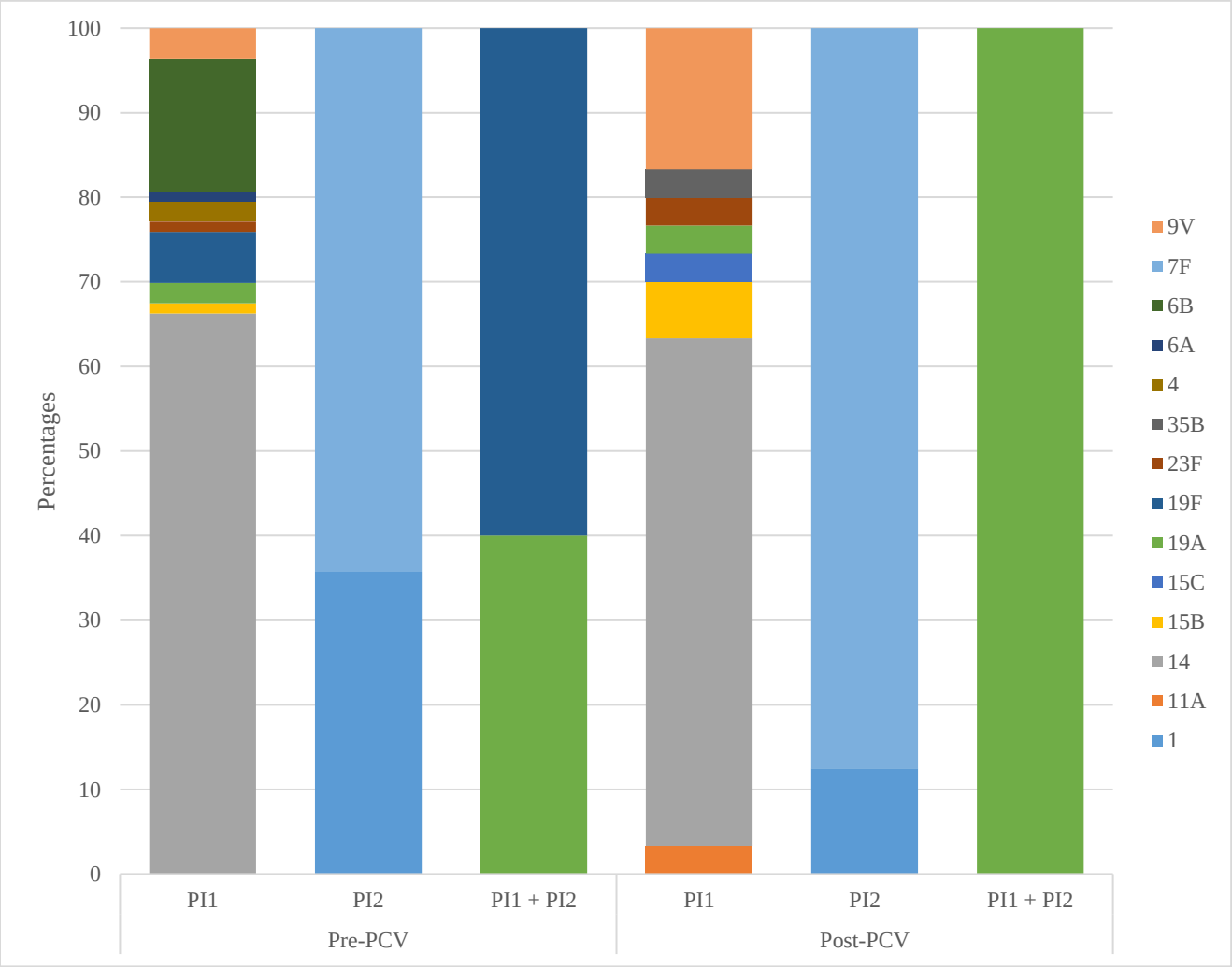

Figure 9: Serotype Distribution with Positive Pilus and Presence Pre-and Post-PCV